Role of Interleukin-10 in Regulation of T-Cell-Dependent and T-Cell-Independent Mechanisms of Resistance to *Toxoplasma gondii*

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Interleukin-10 (IL-10) is a cytokine which can inhibit T-cell and natural killer (NK) cell functions associated with cell-mediated immunity to intracellular infections. The production of IL-10 by mice infected with *Toxoplasma gondii* has been implicated in the suppression of lymphocyte proliferation observed during acute toxoplasmosis, as well as susceptibility to infection with this parasite. We have used C57BL/6 mice which lack a functional IL-10 gene (IL-10^{-/-} mice) to investigate the role of IL-10 in acute toxoplasmosis. Intraperitoneal infection of IL-10^{-/-} mice with *T. gondii* resulted in 100% mortality by day 13, whereas wild-type C57BL/6 (WT) mice survived acute infection. IL-10^{-/-} mice infected with *T. gondii* had significantly higher serum levels of IL-12 and gamma interferon (IFN- γ) than WT mice. Early mortality of infected IL-10^{-/-} mice was prevented by treatment with IL-10 and significantly delayed by neutralizing antibodies to IL-12 and IFN- γ . Further studies revealed that SCID/IL-10^{-/-} mice infected with *T. gondii* had delayed time to death compared to IL-10^{-/-} mice, indicating that lymphocytes contributed to death of IL-10^{-/-} mice. In addition, infected SCID/IL-10^{-/-} mice survived longer than infected SCID mice. These latter data indicate that in mice lacking lymphocytes, endogenous IL-10 is associated with increased susceptibility to *T. gondii*. However, the lack of IL-10 does not alter the infection-induced suppression of T cell and NK cell functions. Our experiments reveal that IL-10 is associated with protection or increased susceptibility to infection with *T. gondii*, depending on whether mice possess lymphocytes, and demonstrate the important roles of IL-12 and IFN- γ in the early infection-induced mortality observed in the IL-10^{-/-} mice.

Interleukin 10 (IL-10) is a cytokine with pleiotropic effects on cells of the immune system and is commonly associated with inhibition of cell-mediated immune responses. In particular, IL-10 appears to negatively regulate T-cell and natural killer (NK) cell functions (13, 36). Specifically, IL-10 is able to inhibit T-cell and NK cell production of gamma interferon (IFN- γ) by suppressing macrophage production of cytokines (IL-1, tumor necrosis factor alpha [TNF- α], and IL-12) required to stimulate production of IFN- γ (2, 13). Moreover, IL-10 can decrease macrophage expression of major histocompatibility complex class II and B7 molecules and thereby inhibit antigen presentation and costimulatory functions of these cells (13, 14). Other effects of IL-10 include its ability to inhibit macrophage production of nitric oxide which results in impaired killing of intracellular organisms (13, 17). Because of these inhibitory effects, it has been proposed that IL-10 may down-regulate protective immune responses to several intracellular pathogens, such as *Mycobacterium avium*, *Leishmania* sp., *Trypanosoma cruzi*, and *Toxoplasma gondii* (12, 19, 24, 25, 26, 42).

During acute toxoplasmosis, there is a suppression of in vitro lymphocyte proliferation to mitogens and, more importantly, to *T. gondii* lysate antigen (TLA) (9, 10, 35). It has been hypothesized that this temporary suppression gives *T. gondii* an advantage in establishing a chronic disease within the host, and

similar suppressive responses have been associated with other infectious diseases (1, 37, 39, 41). Previous work from this laboratory demonstrated that IFN- γ -induced production of reactive nitrogen intermediates (RNI) is involved in mediating the suppression of lymphocyte proliferation to concanavalin A (ConA) and TLA (7, 8). However, other investigators have reported that this suppression is due largely to IL-10 (31). Acute toxoplasmosis is also associated with an initial increase in NK cell activity which is suppressed within 1 week after infection (23). Collectively, these findings suggest that chronic disease following infection with *T. gondii* may be the result of suppressed T-cell functions as well as suppressed NK cell functions.

We wanted to determine whether C57BL/6 mice which were deficient in IL-10 by gene mutation (IL-10^{-/-} mice [3, 32]) still exhibited suppressed in vitro lymphocyte proliferation to mitogen and TLA and whether NK cell activity would be altered in these mice. In initial experiments, infection of IL-10^{-/-} and wild-type C57BL/6 (WT) mice intraperitoneally (i.p.) with *T. gondii* revealed that IL-10^{-/-} mice succumbed to infection significantly earlier than WT mice. We performed experiments to characterize the immune response in IL-10^{-/-} mice by analyzing the role of cytokines and lymphocytes in the early mortality. Subsequently, the proliferative capacities and cytolytic activities of IL-10^{-/-} spleen cells were assessed and compared to the responses of WT spleen cells.

MATERIALS AND METHODS

***T. gondii* strains.** Cysts of strain ME49 were prepared from brains of donor mice as previously described (5, 6). Mice were infected with 20 cysts by i.p.

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injection in a volume of 0.2 ml. Strain RH tachyzoites were harvested from the peritoneal fluid of Swiss Webster mice that had been injected with tachyzoites 2 to 3 days earlier (47). TLA was prepared from strain RH tachyzoites as previously described (40). TLA was titrated to determine the optimal concentration for splenocyte proliferation and was used at 40 μ g/ml for these experiments.

Mice. Female Swiss Webster mice and C57BL/6 mice were obtained from Simonsen Laboratories (Gilroy, Calif.) or B and K Universal, Inc. (Fremont, Calif.). IL-10^{-/-} mice were provided by DNAX, Palo Alto, Calif. (3). These mice were generated by backcrossing C57BL/6-129/Ola IL-10^{-/-} mice (32) onto the C57BL/6 background for seven generations (3). The C57BL/6 IL-10^{-/-} mice were less susceptible to inflammatory bowel disease than the C57BL/6-129/Ola IL-10^{-/-} mice and appeared to be healthy for at least 4 months. IL-10^{-/-} mice backcrossed onto a C57BL/6-SCID background (SCID/IL-10^{-/-} mice) were also used. C57BL/6-SCID mice were used as controls. SCID mice were housed in cages within laminar-flow racks with sterile food and water. Each experimental group contained three to five mice between 6 and 14 weeks of age.

Histology. At the time of sacrifice, samples of lung, liver, and heart were removed from each mouse, fixed in 4% formaldehyde–70% ethanol–0.8 N acetic acid, and embedded in paraffin as previously described (26). Organs were sectioned and stained with hematoxylin and eosin for visualization of pathological changes. *T. gondii* parasites and antigens were detected in livers of infected mice by peroxidase-antiperoxidase staining using polyclonal rabbit antibodies against *T. gondii* (or against *Trypanosoma cruzi* as a negative control) as previously described (11). Liver samples from Swiss Webster mice infected with the RH strain *T. gondii* for 3 days were used as positive controls.

Reagents. Complete RPMI 1640 (Gibco BRL, Life Technologies, Gaithersburg, Md.) medium contained 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, Utah) and penicillin (100 U/ml), streptomycin (100 μ g/ml), and amphotericin B (Fungizone; 0.25 μ g/ml) (BioWhittaker, Walkersville, Md.). *N*⁶-Monomethyl-L-arginine monoacetate salt (NMMA; Calbiochem-Novabiochem, La Jolla, Calif.) was reconstituted at 10.5 mM in complete medium and stored at –80°C until use at a final concentration of 0.5 mM. ConA (Sigma, St. Louis, Mo.) was titrated to determine the optimal concentration for splenocyte proliferation and used at a final concentration of 2.5 μ g/ml. Neutralizing monoclonal antibody (MAb) specific for IFN- γ (XMG1.2; rat immunoglobulin G1 [IgG1]; used in vitro at 120 μ g/ml; used in vivo at 2, 0.5, or 0.12 mg/mouse injected i.p. 5 days postinfection or 4 mg injected i.p. on days 5 and 7 postinfection) and control MAb (GL113; anti- β -galactosidase; rat IgG1) were supplied by John Abrams, DNAX. Neutralizing antibodies for IL-12 (C17.8; rat IgG2a) were provided by Phillip Scott, University of Pennsylvania, Philadelphia, and administered at 1 mg/mouse i.p. 5 days postinfection, 0.5 mg/mouse 8 days postinfection, and 0.2 mg/mouse 12 days postinfection. Neutralizing MAb specific for TNF- α (XT.22; rat IgG1; 4 mg administered i.p. on days 5 and 7 postinfection) was provided by Yasuhiro Suzuki, Palo Alto Medical Foundation, Palo Alto, Calif. Recombinant murine IL-10 was prepared as described previously (4), and 12 μ g/mouse/day was injected i.p. from days 5 to 15 postinfection. Previous studies had demonstrated that 10 μ g of IL-10 administered i.p. to IL-10^{-/-} mice protected them from lipopolysaccharide-induced endotoxic shock (4).

Cytokine enzyme-linked immunosorbent assay. IFN- γ and IL-4 levels were measured by using a two-site enzyme-linked immunosorbent assay as previously described (38). Reagents were supplied by John Abrams. TNF- α was measured by using MAb MP6-XT22 and biotinylated MAb MP6-XT3 (Pharmingen, San Diego, Calif.) according to the manufacturer's instructions. IL-12 (p40) levels were measured by using MAb C17.8 and biotinylated C15.6 (MAbs provided by Dale Umetsu, Stanford University, Stanford, Calif.) according to the Pharmingen protocol.

Splenocyte proliferation assay. Splenocyte cultures were prepared as previously described (7). Briefly, spleens were disrupted to obtain a single-cell suspension, followed by lysis of erythrocytes in 0.83% NH₄Cl–0.01 M Tris-HCl. The remaining splenocytes were washed and plated in 96-well flat-bottom plates at 4 $\times$ 10⁵ cells per well. Cells were incubated with complete medium, ConA, or TLA, and other additions where noted, in triplicate wells in a final volume of 200 μ l. Plates were incubated at 37°C in 5% CO₂ for 48 h (ConA samples) or 96 h (TLA samples). Proliferation of T lymphocytes was assessed by pulsing splenocyte cultures with 1 μ Ci of [³H]thymidine ([³H]TdR; 1 mCi/ml; Amersham, Chicago, Ill.) for an additional 18 to 24 h. Cells were harvested with a PHD cell harvester (Cambridge Technology, Inc., Watertown, Mass.), and incorporation of radioactivity was determined with a scintillation counter (LS 6500; Beckman Instruments, Fullerton, Calif.). Forty-eight-hour culture supernatants were used for cytokine or nitrite analysis.

Cytofluorometric analysis. Splenocytes were isolated as described above and instead of being cultured were washed in a buffer consisting of 140 mM NaCl, 5 mM KCl, 1 mM NaH₂PO₄ · 7H₂O, 0.4 mM KH₂PO₄, 5.5 mM glucose, 1 mM azide, and 0.1% bovine serum albumin and were resuspended in 0.2 ml. One microliter of appropriate antibody (fluorescein isothiocyanate- or phycoerythrin-labeled anti-mouse CD8a, anti-mouse CD4, or anti-mouse NK cells [Pharmingen]; rat MAb to mouse macrophage F4/80 [Caltag Laboratories, South San Francisco, Calif.]) was added to each tube, and cells were incubated on ice for 30 min. Cells were washed in excess buffer and resuspended in 1% paraformaldehyde prior to analyzing on a FACScan cytofluorometer (Becton Dickinson and Company, San Jose, Calif.). Background fluorescence was measured by using

FIG. 1. Effect of infection with *T. gondii* on survival of IL-10^{-/-} mice. WT ($n = 5$) or IL-10^{-/-} ($n = 18$) (data pooled from three separate experiments) mice were infected i.p. with 20 cysts of *T. gondii* ME49.

fluorescein isothiocyanate- or phycoerythrin-labeled rat IgG antibodies (Pharmingen).

Nitrite production. Levels of nitrite in cultures of splenocytes were measured by the Greiss assay (21). Fifty-microliter aliquots of culture supernatant samples plus 50 μ l of a 1:1 mixture of 0.1% naphthylethylenediamide dihydrochloride (Sigma) in 2.5% H₃PO₄ and 1% sulfanilamide (Sigma) in 2.5% H₃PO₄ were added to 96-well flat-bottom plates and incubated at room temperature for 10 to 20 min, and absorbances were read at 550 nm. Standard curves were prepared by using sodium nitrite (Sigma) from 0.2 to 50 nmol/ml.

NK cell assays. Cytotoxicity of YAC-1 cells was used as a measure of NK cell activity (22, 27, 29, 44, 46). YAC-1 targets were labeled with Na⁵¹Cr O₄ and incubated at a spleen cell effector target ratio of 100:1 as previously described (27, 44). Supernatants were measured for ⁵¹Cr and used to estimate specific cytolytic activity. Levels of radioactivity were measured with a Beckman LS6500 scintillation counter. Data are expressed as the means of triplicate wells.

Statistics. INSTAT software (GraphPad, San Diego, Calif.) was used for unpaired two-tailed Student *t* test or Welch test evaluations. *P* < 0.05 was considered significant. For survival curves, *P* values were determined by using survival tools in StatView (Abacus Concepts, Berkeley, Calif.).

RESULTS

Effect of absence of IL-10 on survival of mice infected with *T. gondii*. Initial experiments designed to assess lymphocyte proliferation in infected IL-10^{-/-} mice revealed that IL-10^{-/-} mice were more susceptible than WT mice to infection with *T. gondii*. Following i.p. infection with 20 cysts of *T. gondii*, all WT mice survived the acute phase of the infection, whereas all IL-10^{-/-} mice died within 13 days (Fig. 1). As expected, all WT mice died between 10 and 14 weeks postinfection as a consequence of toxoplasmic encephalitis (data not shown).

Histopathological examination of the hearts, livers, and lungs of WT and IL-10^{-/-} mice 7 days postinfection revealed few inflammatory foci in each of these organs; a larger number of inflammatory foci were noted in livers of IL-10^{-/-} mice than in those of WT mice. However, by 9 days after infection, the pathology in the livers of IL-10^{-/-} mice was more severe, with large foci of inflammation and areas of necrosis, compared to livers from WT mice (data not shown). Liver sections from WT or IL-10^{-/-} mice infected for 9 days were stained for the presence of *T. gondii* by using peroxidase-antiperoxidase techniques. Significant staining was not observed in any of the samples (data not shown). Thus, IL-10^{-/-} mice did not appear to die of an overwhelming infection.

FIG. 2. Effect of absence of IL-10 on survival of SCID mice. SCID ($n = 6$; data pooled from two separate experiments) and SCID/IL-10^{-/-} ($n = 6$; data pooled from two separate experiments) mice were infected i.p. with 20 cysts of strain ME49. Survival of IL-10^{-/-} mice ($n = 6$) infected with 20 ME49 cysts in a separate experiment is shown for comparison.

Role of lymphocytes in mortality of IL-10^{-/-} mice infected with *T. gondii*. SCID/IL-10^{-/-} mice (on a C57BL/6 background) were used to assess whether lymphocytes contributed to the early mortality observed in infected IL-10^{-/-} mice (also on a C57BL/6 background). The Kaplan-Meier product limited survival analysis revealed that infected SCID/IL-10^{-/-} mice survived significantly longer than infected IL-10^{-/-} mice, surviving past the acute phase of infection (Fig. 2; $P = 0.0007$, log rank test). At the time of death, histopathological examination of the organs of SCID/IL-10^{-/-} mice revealed that they had large numbers of parasites within the brain and foci of cellular infiltrates in the liver (data not shown).

SCID/IL-10^{-/-} mice also survived significantly longer than infected SCID mice (Fig. 2; $P = 0.0006$, log rank test). As expected, histopathological examination of organs from SCID mice infected with *T. gondii* showed evidence of an overwhelming parasite burden at the time of death (data not shown).

Roles of IFN- γ and IL-12 in mortality of IL-10^{-/-} mice infected with *T. gondii*. To assess how the immune response in infected IL-10^{-/-} mice differed from that of infected WT mice, serum levels of IFN- γ , IL-12, and TNF- α were measured. Serum levels of IFN- γ and IL-12 were significantly higher in IL-10^{-/-} mice than in WT mice beginning at day 5 postinfection and continuing through day 9 postinfection (Table 1). In WT mice, the levels of IFN- γ and IL-12 peaked at day 7 and started to decline by day 9 postinfection. Remarkably, in IL-10^{-/-} mice, IFN- γ levels were still increasing at day 9 and IL-12 levels appeared to plateau. TNF- α was not detectable in these serum samples (detection limit, 1,200 pg/ml).

Spleen cells from WT or IL-10^{-/-} mice infected for 9 days were stimulated in vitro with TLA, and levels of IFN- γ and IL-12 were measured in culture supernatants. IL-10^{-/-} splenocytes released increased levels of IFN- γ either spontaneously (IL-10^{-/-}, 8.9 ± 1.1 ng/ml; WT, ≤ 0.1 ng/ml; $P = 0.004$; data expressed as means $\pm$ standard deviations [SD] from triplicate culture wells) or after TLA stimulation (IL-10^{-/-}, 139.6 ± 3.4 ng/ml; WT, 76.5 ± 2.1 ng/ml; $P < 0.0001$). In addition, IL-10^{-/-} splenocytes produced greater levels of IL-12 after TLA stimulation (IL-10^{-/-}, 16.0 ± 0.9 ng/ml; WT, 3.8 ± 0.1 ng/ml; $P = 0.002$). IL-4 was not detected in culture supernatants (detection limit, 80 pg/ml).

To determine whether the high levels of IFN- γ and/or IL-12 contributed to the early death of infected IL-10^{-/-} mice, neutralizing antibodies to these cytokines were administered to infected IL-10^{-/-} mice. Because these cytokines are crucial for protection against *T. gondii* (16, 45), administration of anti-IL-12 or anti-IFN- γ antibody was initiated 5 days after infection, in order to avoid depriving mice of these cytokines during early infection. Anti-IL-12 MAb significantly delayed time to death of infected IL-10^{-/-} mice compared to controls (Fig. 3; $P = 0.0001$, log rank test). Administration of anti-IFN- γ MAb also significantly delayed time to death (Fig. 3; $P < 0.0001$, log rank test). Noteworthy is that at day 12 postinfection, 90% of control IL-10^{-/-} mice had died, whereas all IL-10^{-/-} mice treated with anti-IL-12 or anti-IFN- γ were alive. There was no significant difference in the mortality of IL-10^{-/-} mice treated with anti-IFN- γ or anti-IL-12 MAb ($P = 0.28$, log rank test). In a single experiment, we treated infected IL-10^{-/-} mice with a combination of anti-IL-12 and anti-IFN- γ MAbs (using identical amounts and following the same time schedule used for individual treatments). This regimen did not afford protection beyond that shown by the antibodies separately (data not shown). Administration of neutralizing MAb for TNF- α (4 mg on days 5 and day 7 postinfection) did not alter mortality of infected IL-10^{-/-} mice (data not shown). These data indicate that IL-12 and IFN- γ contribute to the early mortality of IL-10^{-/-} mice infected with *T. gondii*.

To determine whether survival of IL-10^{-/-} mice could be extended beyond what was observed in the neutralizing antibody studies, we administered exogenous IL-10 to infected IL-10^{-/-} mice. Administration of 12 μ g of IL-10 i.p. began on day 5 postinfection and continued daily for 10 days. This treatment conferred significant protection to IL-10^{-/-} mice, allowing 71% to survive for 30 days after infection (Fig. 3; $P = 0.001$, log rank test for survival compared to control IL-10^{-/-} mice). Nevertheless, these mice died by day 46 postinfection (data not shown). Examination of the organs of these mice at the time of death revealed large numbers of parasites within the brain and foci of inflammatory cells in the liver, with no apparent necrosis of the liver (data not shown). Interestingly, at 9 days postinfection, serum levels of IFN- γ and IL-12 in IL-10^{-/-} mice

TABLE 1. Serum levels of IFN- γ and IL-12 in IL-10^{-/-} and WT mice following infection with *T. gondii*

Mice	IFN- γ (pg/ml)				IL-12 (pg/ml)			
	Day 0 ^a	Day 5 ^a	Day 7 ^a	Day 9 ^b	Day 0 ^a	Day 5 ^a	Day 7 ^a	Day 9 ^b
IL-10 ^{-/-}	≤ 160	$4,568 \pm 1,694^c$	$21,408 \pm 2,244^d$	$59,072 \pm 20,426^e$	$2,329 \pm 1,851$	$8,563 \pm 2,451^c$	$20,304 \pm 4,842^d$	$20,149 \pm 4,942^e$
WT	≤ 160	$1,928 \pm 924$	$12,266 \pm 4,258$	$7,056 \pm 7,292$	$1,013 \pm 693$	$4,377 \pm 2,468$	$8,675 \pm 5,361$	$5,908 \pm 4,803$

^a Mean $\pm$ SD for five to seven mice.

^b Mean $\pm$ SD for 9 to 12 mice.

^c Significantly different from WT value; $P \leq 0.01$.

^d Significantly different from WT value; $P \leq 0.002$.

^e Significantly different from WT value; $P \leq 0.0001$.

FIG. 3. Effect of anti-IL-12 MAb, anti-IFN- γ MAb, and exogenous IL-10 on survival of IL-10^{-/-} mice. IL-10^{-/-} mice were infected i.p. with 20 cysts of strain ME49. Five days later, anti-IL-12 MAb ($n = 7$), anti-IFN- γ MAb ($n = 9$), or IL-10 ($n = 7$) was administered (anti-IL-12, 1 mg on day 5 postinfection, 0.5 mg on day 8, and 0.2 mg on day 12; anti-IFN- γ , 4 mg on day 5 and 4 mg on day 7; IL-10, 12 μ g/day from days 5 to 15 postinfection). Data are pooled from two separate experiments for each treatment. Control IL-10^{-/-} mice were infected and 5 days later given anti- β -galactosidase antibodies or phosphate-buffered saline in the same manner as treated mice; data were pooled for these control treatments ($n = 12$). Giving a single dose of either 0.12, 0.50, or 2.0 mg of anti-IFN- γ MAb 5 days postinfection did not significantly delay time to death (data not shown).

treated with IL-10 were not significantly different from the high levels of these cytokines observed in control treated IL-10^{-/-} mice [IFN- γ , $57,673 \pm 32,030$ pg/ml for control IL-10^{-/-} mice and $70,010 \pm 57,310$ pg/ml for IL-10-treated IL-10^{-/-} mice [$P = 0.6$, $n = 7$ per group]; IL-12, $17,640 \pm 6,441$ pg/ml for control IL-10^{-/-} mice and $16,500 \pm 5,643$ pg/ml for IL-10-treated IL-10^{-/-} mice [$P = 0.3$; $n = 7$]). However, by 12 days postinfection, there was a remarkable reduction in the serum levels of both IFN- γ and IL-12 in IL-10-treated IL-10^{-/-} mice (IFN- γ , $1,793 \pm 1,850$ pg/ml; IL-12, $894 \pm 1,080$ pg/ml; $n = 12$). Although control IL-10^{-/-} mice had died or were ill 12 days postinfection, serum levels of IFN- γ and IL-12 measured in three mice at this time point were also low (IFN- γ , $2,080 \pm 865$ pg/ml; IL-12, $2,090 \pm 1,020$ pg/ml).

We determined whether the increased levels of IFN- γ and IL-12 in IL-10^{-/-} mice correlated with differences in the percentages of CD4⁺, CD8⁺, NK1.1⁺, or F4/80⁺ (a macrophage marker) cells from spleens of infected IL-10^{-/-} mice compared to WT mice. Cytofluorometry was used to analyze these cell subsets within the spleens of IL-10^{-/-} and WT mice infected for 9 days. No significant differences in the percentages of these cell subsets in IL-10^{-/-} or WT mice were demonstrable; in addition, there were no differences in total spleen cell numbers between infected IL-10^{-/-} and WT mice (data not shown). The percentages of CD4⁺ and CD8⁺ cells from the mesenteric lymph nodes and peritoneal cavities of infected IL-10^{-/-} and WT mice were also equivalent (data not shown).

Infection induced suppression of NK cell and T-cell function in IL-10^{-/-} mice. During the acute phase of infection of mice with *T. gondii*, NK cell cytolytic activity is increased dramatically by day 3 postinfection but becomes suppressed by day 7 (23). Since IL-10 is a potent inhibitor of NK cell functions and of NK cell-dependent resistance to *T. gondii* in SCID mice (13,

26), we determined whether the lack of endogenous IL-10 production would affect NK cell activity in infected mice. Lysis of the YAC-1 target cell has previously been used by this laboratory, and by others, as a measure of infection-induced increases in NK cell activity (22, 27, 29, 43, 46). NK cytolytic activity of IL-10^{-/-} splenocytes increased, peaked at day 5, and returned to baseline levels by day 7 postinfection (Fig. 4). Similar results were observed with WT splenocytes (Fig. 4), in agreement with previously described studies from our laboratory (23). These data show that the absence of IL-10 does not affect the kinetics of NK cell cytolytic activity in response to infection.

IL-10 has been implicated as having a role in the suppression of in vitro lymphocyte proliferation to mitogen and TLA during acute toxoplasmosis (7, 8). To assess how a lack of endogenous IL-10 would affect this suppression, we performed a series of experiments in which splenocytes from either uninfected or infected WT or IL-10^{-/-} mice were cultured with ConA or TLA, and T-cell proliferation was assessed by [³H]TdR incorporation. Splenocytes from IL-10^{-/-} and WT mice showed reduced proliferation in response to ConA on day 5 postinfection compared to uninfected controls and marked suppression on day 7 of infection (Fig. 5A). There was no statistical difference between results obtained with splenocytes from infected WT and IL-10^{-/-} mice. Similar results were obtained when TLA was used instead of ConA, with markedly decreased proliferation on day 7 postinfection compared to day 5 postinfection (Fig. 5B).

Effects of NMMA and anti-IFN- γ antibody on suppressed lymphocyte proliferation in infected IL-10^{-/-} mice. We wanted to assess whether the lymphocytes from infected IL-10^{-/-} mice showed a suppressed proliferative response to in vitro stimulation with ConA because of IFN- γ -induced production of RNI. This mechanism has been shown to mediate suppression of lymphocyte proliferation in normal mice (7, 31). Addition of NMMA, an inhibitor of RNI production, or a neutralizing MAb specific for IFN- γ to ConA-stimulated cultures of splenocytes from infected WT or IL-10^{-/-} mice reversed the suppression (Fig. 6). Although the addition of anti-

FIG. 4. Analysis of cytotoxic activity of NK cells from *T. gondii*-infected WT and IL-10^{-/-} mice. Splenocytes from WT or IL-10^{-/-} mice infected for 0, 3, 5, or 7 days were used as effector cells in a standard ⁵¹Cr release assay. The effector/target ratio was 100:1. Splenocytes were pooled from three mice for each time point. Similar results were observed in two repeat experiments.

FIG. 5. Analysis of lymphocyte proliferative responses in WT and IL-10^{-/-} mice infected with *T. gondii*. (A) Splenocytes from uninfected or infected mice were cultured with ConA and then pulsed with [³H]TdR. Proliferation is shown for IL-10^{-/-} (solid bars) and WT (hatched bars) splenocytes. Splenocytes were pooled from three mice for each time point. Error bars represent the SD from triplicate culture wells. These results are representative of four separate experiments. *, significantly different from day 0 values (IL-10^{-/-} $P = 0.046$; WT $P = 0.035$). **, significantly different from day 5 values (IL-10^{-/-} $P = 0.003$; WT $P = 0.012$) and day 0 values (IL-10^{-/-} $P = 0.0095$; WT $P = 0.015$). (B) Splenocytes were cultured with TLA, and proliferation was assessed as for panel A. Results are representative of four separate experiments. *, significantly different from day 0 IL-10^{-/-} value ($P = 0.05$). **, significantly different from day 5 values (IL-10^{-/-} $P = 0.0009$; WT $P = 0.0005$) and day 0 values (IL-10^{-/-} $P = 0.036$; WT $P = 0.043$).

IFN- γ MAb resulted in greater proliferation than did addition of NMMA (as previously reported by Candolfi and coworkers [7]), there were no differences between levels of [³H]TdR uptake for IL-10^{-/-} and WT mice. Similarly, in splenocyte cultures incubated with TLA, the low proliferation observed 7 days postinfection (Fig. 5B) was increased upon addition of NMMA or anti-IFN- γ MAb (data not shown). These results support our earlier findings that IFN- γ -induced production of RNI mediates, in part, the infection-induced suppression of lymphocyte proliferation (7, 8) and extends these results to show that this mechanism of suppression is functional in the absence of IL-10.

To determine whether there were differences in nitrite production (as a measure of RNI) between infected IL-10^{-/-} and WT mice, levels of nitrite were measured from supernatants of ConA-stimulated spleen cell cultures from IL-10^{-/-} or WT mice infected for 7 days. No differences were observed (IL-10^{-/-} mice, 12.8 ± 2.3 nmol/ml; WT mice, 12.3 ± 1.2 ; data expressed as means $\pm$ standard errors of the means from four separate experiments). Similarly, no differences in nitrite production were measured from supernatants of TLA-stimulated spleen cell cultures from IL-10^{-/-} or WT mice infected for 7 days (IL-10^{-/-} mice, 10.1 ± 1.9 nmol/ml; WT mice, 9.5 ± 1.2 ; means $\pm$ standard errors of the means from four separate experiments). Interestingly, splenocytes from uninfected IL-10^{-/-} mice stimulated with ConA produced higher levels of nitrite than splenocytes from uninfected WT mice (3.03 ± 1.36 nmol/ml versus 1.23 ± 0.45 nmol/ml; data pooled from four separate experiments; $P = 0.046$).

DISCUSSION

Our aim was to determine whether IL-10 was critical for the suppression of lymphocyte proliferation to mitogen and TLA which occurs during acute toxoplasmosis. During our first set of experiments, it was observed that i.p. infection of IL-10^{-/-} mice with 20 cysts of *T. gondii* ME49 resulted in early mortality compared to WT controls. IL-10^{-/-} mice did not survive the

acute phase of infection and died within 2 weeks, whereas WT mice survived for 10 to 14 weeks but eventually died of toxoplasmic encephalitis. The early mortality of IL-10^{-/-} mice was correlated with higher serum levels of IFN- γ and IL-12 than in WT mice. However, there was no correlation between increased cytokine levels and the percentages of CD8⁺, CD4⁺, NK1.1⁺, or F4/80⁺ cells in spleens of infected IL-10^{-/-} and WT mice. Histopathological evaluation of the organs of infected IL-10^{-/-} mice indicated that the livers were necrotic, contained large numbers of foci of inflammatory cell infiltrates

FIG. 6. Effect of NMMA or anti-IFN- γ MAb on spleen cell proliferation. Splenocytes from IL-10^{-/-} (solid bars) or WT (hatched bars) mice infected for 7 days were cultured with ConA alone, ConA plus NMMA, or ConA plus anti-IFN- γ , and [³H]TdR incorporation was measured. Counts for untreated IL-10^{-/-} mice = $1,338 \pm 1,288$; counts for untreated WT mice = 601 ± 179 . Splenocytes were pooled from three mice for each treatment. The data are expressed as the means from three separate experiments, with error bars indicating the SD. *, significantly different from NMMA values (IL-10^{-/-} $P = 0.01$; WT $P = 0.01$) and from anti-IFN- γ values (IL-10^{-/-} $P = 0.02$; WT $P = 0.01$). **, significantly different from anti-IFN- γ value (WT $P = 0.03$).

by 9 days after infection, but contained few parasites. Livers from similarly infected WT mice contained fewer inflammatory foci and showed no evidence of necrosis. Thus, although *T. gondii* is able to trigger a host immune response in the liver, the continued presence of large numbers of parasites does not appear necessary to mediate the pathologic effects observed. Of relevance to these results is that administration of IL-12 to normal mice results in focal mononuclear cell infiltrates as well as necrotic foci in the liver, noted by Gately and colleagues (15).

In agreement with the studies presented here, Gazzinelli and colleagues recently reported that IL-10^{-/-} mice infected with *T. gondii* die early in infection, show elevated levels of serum IL-12 and IFN- γ , and have no demonstrable increase in parasite burden (18). In addition, these authors showed that macrophages from IL-10^{-/-} mice produced higher levels of TNF- α and IL-12 after stimulation with *T. gondii* compared to macrophages from WT mice. These investigators implied that IL-10^{-/-} mice succumb to infection with *T. gondii* due to high levels of inflammatory cytokines, but direct evidence was lacking. However, our results show directly that IL-12 and IFN- γ contributed to the early death of these mice. Given the critical role of both of these cytokines in protection against *T. gondii* (16, 45), it is remarkable that neutralization of either of these cytokines could protect any mouse strain infected with *T. gondii*. Interestingly, a recent report by Liesenfeld et al. (33) revealed that C57BL/6 mice, which are susceptible to peroral infection with *T. gondii* and show severe necrosis in the ilea after infection, had prolonged survival and reduced necrosis compared to control mice if given anti-IFN- γ MAb. Thus, our results and those of Liesenfeld et al. (33) demonstrate the fine distinction between a protective and pathogenic immune response to infection. In our model, we have demonstrated the critical role of IL-10 in regulating these responses. In another study, Linke and colleagues reported that IL-10^{-/-} mice infected with *Plasmodium chabaudi* died earlier than controls, most likely due to a lethal inflammatory response rather than fulminating parasitemia (34). Thus, it appears that the role of IL-10 in preventing infection-induced immune hyperreactivity is not restricted to *T. gondii* and may be a general principle related to other infections.

Administration of recombinant murine IL-10 to infected IL-10^{-/-} mice significantly delayed time to death. Analysis of the brains of IL-10-treated IL-10^{-/-} mice at the time of death revealed the presence of *T. gondii* cysts, indicating that a chronic infection had been established. These results suggest that the early mortality of infected IL-10^{-/-} mice is most likely due to a direct effect of a lack of IL-10 and not some other unknown defect in the immune system of IL-10^{-/-} mice. In addition, these results demonstrate that it is possible to protect IL-10^{-/-} mice through the acute phase, allowing a chronic infection to develop. IL-10 treatment afforded dramatic protection to infected IL-10^{-/-} mice, beyond that seen with administration of anti-IFN- γ or anti-IL-12 antibody. Thus, these data suggest that neither IL-12 nor IFN- γ is solely responsible for the early mortality of IL-10^{-/-} mice. Interestingly, treatment with IL-10 did not significantly alter serum levels of IFN- γ and IL-12 in infected IL-10^{-/-} mice. However, since there was a significant difference in the time to death of these mice compared to infected control IL-10^{-/-} mice, our results suggest that exogenous IL-10 has other modulating effects on the immune system of IL-10^{-/-} mice which are not revealed simply by measuring serum IFN- γ and IL-12. Taken together, our results show a role for IFN- γ and IL-12 in contributing to the early mortality of infected IL-10^{-/-} mice, but other factors may also play a part in this process. IL-10 is known to have

pleiotropic effects on the immune system, such as decreasing macrophage production of IL-1 and IL-6, inhibiting expression of major histocompatibility complex class II and B7 molecules, and inhibiting macrophage production of nitric oxide (13, 14, 17). Altered regulation of these events in IL-10^{-/-} mice might also contribute to their early mortality after infection with *T. gondii*.

Following infection with *T. gondii*, SCID/IL-10^{-/-} mice survived significantly longer than IL-10^{-/-} mice, indicating that lymphocytes play a role in the early mortality of IL-10^{-/-} mice. This outcome is consistent with the recent findings by Gazzinelli and colleagues, who reported that depletion of CD4⁺ T cells from IL-10^{-/-} mice resulted in delayed time to death (18). Our data also demonstrate that IL-10 is associated with greater susceptibility of SCID mice to *T. gondii*, since SCID/IL-10^{-/-} mice survived infection for a significantly longer time than SCID mice. SCID mice normally survive 15 to 23 days after infection with *T. gondii* (26–28) due to a T-cell-independent protective response mediated by IFN- γ production from NK cells, but these mice ultimately cannot control parasite replication without lymphocytes (26, 28). IL-10 has been shown to down-regulate the protective IFN- γ production in SCID mice: in previous studies from our laboratory, treatment of SCID mice with neutralizing antibodies to IL-10 resulted in a delay in time to death compared to control SCID mice (26). The result reported here for SCID/IL-10^{-/-} mice confirms this result and extends it to the natural situation without the necessity of administration of exogenous antibody. Thus, our data indicate that IL-10 production in immunocompetent mice is required to survive infection with *T. gondii*, since mice which lack IL-10 die during the acute phase from a dysregulated immune response. In contrast, in SCID mice, IL-10 production is associated with a greater susceptibility to the parasite-mediated mortality.

Several investigators have proposed a role for IL-10 in the suppression of NK cell function (reviewed in reference 2). The cytolytic activity of NK cells has been used extensively as a measure of NK cell activation during infection with *T. gondii* (20, 27, 29, 44) as well as other parasitic and viral pathogens (22, 43, 46). In acute toxoplasmosis, NK cell activity increases and then decreases within 1 week after infection (23). Our results demonstrate that the absence of IL-10 does not alter the *T. gondii*-induced increase in NK cell activity or the return to baseline levels.

IL-10 has been implicated as an important mediator of the suppression of lymphocyte proliferation which is observed after infection with *T. gondii* (31). Our results demonstrate that proliferation of splenocytes from both IL-10^{-/-} and WT mice to ConA and TLA is reduced at day 7 postinfection. Thus, the presence of IL-10 is not crucial to mediate the suppression of lymphocyte proliferation to mitogen or *T. gondii* antigens. Moreover, our studies demonstrate that IFN- γ -induced RNI production is able to inhibit T-cell proliferation in the absence of IL-10. These data are consistent with our previous results, which demonstrated that IFN- γ -induced RNI was a major mediator of immune suppression in acute toxoplasmosis in normal mice (7, 8). As mentioned above, IL-10 is capable of mediating suppression of lymphocyte proliferation, as demonstrated by Khan et al. (31). Interestingly, when mice which lack the IFN regulatory factor 1 gene and whose macrophages do not produce RNI in response to IFN- γ are infected with *T. gondii*, they still exhibit suppressed lymphocyte proliferation to ConA. These investigators demonstrated that anti-IL-10 antibodies partially restored these proliferative responses. Thus, the down-regulation of lymphocyte proliferation observed during acute toxoplasmosis is unaltered in mice which are unable

to produce IL-10 or unable to produce IFN- γ -induced RNI. This might indicate an overlap in the ability of IL-10 and RNI to inhibit lymphocyte proliferation.

IL-10^{-/-} mice spontaneously develop inflammatory bowel disease, which is most likely due to dysregulated production of cytokines (32). In addition, IL-10 has been shown to be crucial for protection of mice from acute lipopolysaccharide-induced endotoxic shock (4). Thus, it becomes increasingly clear that IL-10 has a role in the down-regulation of cell-mediated immune hyperreactivity. However, our results presented here, in accord with those of Gazzinelli et al. (18), extend these observations to demonstrate the vital importance of IL-10 in down-regulation of infection-induced cytokine production and inflammatory responses. It is hoped that dissection of the function(s) of IL-10 in the regulation of host immune responses during infection will allow a better understanding of the role of IL-10 in the host-parasite relationship as well as in the control of associated immunopathology.

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